Bradykinin receptors in mouse and rat isolated superior cervical ganglia

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1 The ability of bradykinin and its analogues to depolarize rat and mouse superior cervical ganglia was studied by use of *in vitro* grease-gap recording techniques, and the ability of antagonists selective for bradykinin receptor subtypes to block their effects was examined.

2 Bradykinin (3 µM) depolarized ganglia from both species, although the magnitude of the maximal response was less in mouse (15 ± 5%, *n* = 7) than rat tissue (33 ± 6%, *n* = 7), relative to muscarine (1 µM).

3 Interleukin 1β (30 u ml⁻¹ for 18 h at 37°C) increased the depolarization caused by bradykinin (3 µM) in mouse ganglia from 15% to 54% (*P* < 0.001, *n* = 12). Responses to the B₁ receptor agonist, [des-Arg¹⁰]-kallidin (3 µM) were similarly potentiated but this was only detected after inhibition of peptidase activity with 10 µM captopril (4% to 35%, *n* = 5).

4 In ganglia from both species the rank order of agonist potency was bradykinin = [Lys⁰]-bradykinin >> [des-Arg¹⁰]-kallidin. However, like responses to [des-Arg¹⁰]-kallidin in mouse tissue, both the potency of bradykinin and the maximal depolarization achieved (EC₅₀ = 912 nM; 80%, *n* = 11) was enhanced following inhibition of angiotensin converting enzyme with 10 µM captopril (EC₅₀ = 50 nM; 135%, *n* = 4).

5 Responses to bradykinin were selectively antagonized by the B₂ receptor antagonist, Hoe 140 but not by the B₁ antagonist, [Leu⁸]-bradykinin_{1–8}. From Schild analysis the pA₂ value for Hoe 140 in mouse tissue was 9.65, although the slope of the regression line was significantly greater than unity, indicating non-competitive kinetics (slope = 1.88 ± 0.18, *n* = 9). The depolarization caused by [Lys⁰]-bradykinin was also antagonized by Hoe 140 (3 nM).

6 Thus the predominant bradykinin receptor in mouse superior cervical ganglia is compatible with a B₂ subtype. Furthermore the depolarizations caused by B₁ and B₂ agonists in this tissue can be increased following exposure to interleukin 1β, and by blocking peptide degradation with captopril.

Keywords: B₂ bradykinin receptors; superior cervical ganglia; kallidin, Hoe 140; interleukin 1β; electrophysiology

Introduction

Receptors for the nonapeptide bradykinin have been cloned from human, rat and mouse tissues (McEachern *et al.*, 1991; Hess *et al.*, 1992; Eggerikx *et al.*, 1992; McIntyre *et al.*, 1993). These receptors contain seven putative membrane spanning regions and are linked to the activation of phospholipase C, inositol phosphate production, and intracellular calcium mobilization. The existence of at least two types of bradykinin receptors in mammalian tissue, the B₁ and B₂ subtypes, for which antagonists such as Hoe 140 (B₂; Hock *et al.*, 1991; Rhaleb *et al.*, 1992) and the agonist [des-Arg¹⁰]-kallidin (B₁) have different selectivities, has recently been confirmed by cloning of cDNA that encodes the human B₁ receptor (Menke *et al.*, 1994).

In mammals, bradykinin is produced by two pathways; through the direct proteolytic action of kallikreins on high-molecular weight kininogen in plasma following tissue damage, and indirectly by aminopeptidase action on [Lys⁰]-bradykinin (kallidin) which itself is derived from low-molecular weight kininogens. Several of these bradykinin-related peptides are also biologically active. These include [Lys⁰]-bradykinin, and [des-Arg⁹]-bradykinin which is generated via carboxypeptidases that cleave the terminal arginine residue from bradykinin. *In vivo* bradykinin is an efficacious inflammatory agent that has also been implicated in nociception and hyperalgesia (reviewed in Dray & Perkins, 1993). Bradykinin is also a potent vasodilator which increases

vascular permeability and can thus cause hypotension (Regoli & Barabe 1980). The receptor subtype(s) that are involved in the pathophysiological effects of these kinins are not fully understood. B₂ receptors are known to be constitutively expressed by smooth muscle and neuronal tissues and in many, but not all cases, the B₂ receptor ligand Hoe 140 is an effective antagonist. In particular, this includes some of the cardiovascular effects and inflammation caused by bradykinin (Correa & Calixto, 1993). Although in some animal models of neurogenic inflammation (Mantione & Rodriguez, 1990) and nociception (Correa & Calixto, 1993) responses that are insensitive to B₂ antagonists like Hoe 140, and which are more consistent with the activation of B₁ receptors, have been described.

Bradykinin has been implicated as an important peptide neurotransmitter in the sympathetic nervous system (Levine *et al.*, 1986; Lee *et al.*, 1991). However, there have been few direct pharmacological investigations into the receptor subtype(s) present in such tissue. Consequently, we have examined the ability of bradykinin receptor agonists to depolarize isolated superior cervical ganglia, and have compared the ability of selective antagonists to inhibit these responses. Furthermore, because it has been suggested that cloned murine B₂ bradykinin receptors exhibit a mixed B₁/B₂ receptor pharmacology (McIntyre *et al.*, 1993, however *cf.* Hess *et al.*, 1994) we have compared the pharmacology of bradykinin receptor subtypes in both rat and mouse superior cervical ganglia.

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Methods

The methods for dissecting and recording from isolated rodent superior cervical ganglia were the same as described by Seabrook *et al.* (1992). Ganglia were obtained from male Sprague-Dawley rats (150 to 250 g), or from male C57 mice (40 to 60 weeks old), which were then desheathed and placed in a grease-gap recording chamber. The chamber was perfused (1 to 2 ml min⁻¹) with physiological salt solution (PSS) maintained at 25°C and gassed with 95% O₂ and 5% CO₂. The PSS had the following composition (mM): NaCl 125, KCl 5, KH₂PO₄ 1, CaCl₂ 2.5, MgSO₄ 1, NaHCO₃ 25, glucose 10 and tetrodotoxin 0.1 µM. The potential difference between the ganglion cell body (earthed) and the postganglionic trunk was monitored via Ag/AgCl electrodes which were connected via a d.c. amplifier to a chart recorder.

The depolarization caused by bradykinin analogues was normalized with respect to the maximum response obtained with a muscarine under control conditions. Muscarine, 1 µM, was supramaximal in both rat and mouse ganglia. Agonist concentration-effect curves were constructed by semi-cumulative applications of increasing agonist concentrations, the test compound being added to the perfusate for 1 min at 5 min intervals. The appropriate antagonist was then added and following a 60 min equilibration period, the concentration-effect curve was repeated in the continuing presence of the antagonist. The subsequent shifts in the concentration-effect curves were used to construct Schild plots according to the methods of Arunlakshana & Schild (1959), or to estimate the pK_A value (the negative logarithm of the antagonist dissociation constant) using the following equation where the estimated $K_A = -\log [(\text{antagonist concentration})/(\text{concentration ratio} - 1)]$. Concentration-effect curves were fitted to the data by least squares analysis of variance to the equation $Y = Y_{\max}/(1 + (EC_{50}/\text{agonist concentration})^{n_H})$, where the EC₅₀ is the half-maximally effective concentration and n_H is the Hill coefficient, using Grafit (Erithacus Software). Data represent the mean ± s.e. mean, and statistical analysis was carried out using ANOVA (Microsoft Excel ver. 5.0).

Drugs

The following substances were used: bradykinin, [Lys⁰]-bradykinin, bradykinin₁₋₈, [des-Arg¹⁰]-kallidin, Hoe 140 ([D-Arg⁰, Hyp³, β-(2-thienyl)-Ala⁵, D-Tic⁷, Oic⁸]-bradykinin), and [Leu⁸]-bradykinin₁₋₈ were purchased from Bachem; captopril and tetrodotoxin from Sigma; and human interleukin 1β from Boehringer Mannheim. The structure of the bradykinin analogues used in the present study, as well as their synonyms, are shown in Figure 1. Drugs were dissolved in either distilled water or in dimethylsulphoxide (final bath concentration <0.1%) as appropriate, and aliquots of peptide stock solutions were stored at -70°C.

Results

Exogenously applied bradykinin (3 µM) depolarized superior cervical ganglia that had been isolated from both rats and mice. However, relative to the maximum depolarization that

Lys-Arg-Pro-Gly-Phe-Ser-Pro-Phe-Arg-OH	[Lys ⁰]-bradykinin = kallidin
-----Arg-Pro-Gly-Phe-Ser-Pro-Phe-Arg-OH	Bradykinin
-----Arg-Pro-Gly-Phe-Ser-Pro-Phe-----OH	Bradykinin ₁₋₈ =
	[des-Arg ⁹]-bradykinin
Lys-Arg-Pro-Gly-Phe-Ser-Pro-Phe-----OH	[des-Arg ¹⁰] kallidin =
	[des-Arg ¹⁰ -Lys ⁰]-bradykinin
-----Arg-Pro-Gly-Phe-Ser-Pro-Leu-----OH	[Leu ⁸]-bradykinin ₁₋₈

Figure 1 Comparison between the structure of bradykinin analogues used in the present study. Common amino acids are in bold.

Figure 2 Potentiation of the bradykinin-induced depolarization of mouse superior cervical ganglia by pretreatment with interleukin 1β (IL-1β) and captopril. (a) Depolarization caused by maximal concentration of bradykinin in control ganglia (incubated for 18 h at 34°C) and in those that had been exposed to interleukin 1β (30 u ml⁻¹ for 18 h at either 4°C or 37°C). Data are normalized as a percentage depolarization relative to that caused by a supramaximal concentration of muscarine (1 µM). Responses to bradykinin, but not [des-Arg¹⁰]-kallidin ([des-Arg¹⁰]-K), were significantly potentiated by this treatment (**P* < 0.001 compared with control, ANOVA). Data from 5 to 12 ganglia for each treatment. (b) Following exposure to IL-1β (18 h at 37°C), treatment of ganglia with captopril (10 µM) potentiated the depolarization caused by [des-Arg¹⁰]-kallidin (3 µM, *n* = 5). This effect was blocked by the B₁ receptor antagonist, [Leu⁸]-bradykinin₁₋₈ ([Leu⁸]-BK₁₋₈, 10 µM).

was achieved following activation of M₁ muscarine receptors with 1 µM muscarine (e.g. Newberry & Priestley, 1987) the magnitude of the responses was less in mouse (15 ± 5%, *n* = 7) than rat tissue (33 ± 6%, *n* = 7). To examine whether these responses were sensitive to chronic *in vitro* treatment with cytokines, the ability of interleukin 1β (30 u ml⁻¹ for 18 h) to potentiate these depolarizations was studied. In mice, the depolarization caused by bradykinin (3 µM) was increased from 15% to 54 ± 7% (*P* < 0.001, *n* = 12) with IL-1β (Figure 2). This effect was temperature-dependent in that lowering the incubation temperature from 37°C to 4°C precluded the action of IL-1β. In contrast to responses to bradykinin, responses to the B₁ agonist, [des-Arg¹⁰]-kallidin (3 µM) were unaffected by pretreatment with IL-1β (30 u ml⁻¹ for 18 h at 37°C). However, potentiation of responses to [des-Arg¹⁰]-kallidin (3 µM) was observed after inhibition of peptidase activity with 10 µM captopril (4% to 35 ± 11%,

Table 1 Comparison between the potency of bradykinin analogues in rat and mouse isolated superior cervical ganglia, and the affinity of Hoe-140 for receptors activated by bradykinin

	Mouse			Rat		
	pEC ₅₀ /pK _A	nM		pEC ₅₀ /pK _A	nM	
Agonists						
Bradykinin	6.04 ± 0.22	912	(11)	6.77 ± 0.15	170	(7)
[Lys ⁰]-bradykinin	5.89 ± 0.37	1,288	(3)	6.51 ± 0.48	309	(3)
[des-Arg ¹⁰]-kallidin	<5.5	>3,000	(13)	<5.5	>3,000	(3)
[des-Arg ⁹]-bradykinin	<5.0	>10,000	(4)	NT		
Antagonists						
Hoe 140	10.23 ± 0.22	0.06	(3)	10.61 ± 0.16	0.03	(3)
[Leu ⁸]-bradykinin ₁₋₈	<5.0	>10,000	(4)	NT		

Data represent geometric pEC₅₀ values or, as in the case of Hoe 140, the estimated antagonist affinity which was calculated from the rightward shift in the bradykinin concentration-effect curve caused by an antagonist concentration of 3 nM. This estimated pK_A value assumes competitive antagonism which was not observed with Hoe 140, but is included simply as a means of comparing the relative effectiveness of Hoe 140 in mouse and rat ganglia at a known antagonist concentration (see text). Numbers in parentheses represent sample sizes. NT = not tested.

$n = 5$). The depolarization caused by bradykinin was selectively blocked by Hoe 140 (see below), whereas those to [des-Arg¹⁰]-kallidin and not bradykinin were inhibited by [Leu⁸]-bradykinin₁₋₈. The depolarization caused by [des-Arg¹⁰]-kallidin (3 μ M) in the presence of captopril was reduced from 35.4 ± 11.4 ($n = 5$) to $2 \pm 1\%$ after treatment with 10 μ M [Leu⁸]-bradykinin₁₋₈ ($n = 5$).

In ganglia from both rats and mice the rank order of agonist potency was bradykinin = [Lys⁰]-bradykinin >> [des-Arg¹⁰]-kallidin (Table 1, Figure 3). However, like responses to [des-Arg¹⁰]-kallidin the potency and efficacy of bradykinin in mouse tissue ($EC_{50} = 912$ nM; $80 \pm 10\%$, $n = 11$) was significantly increased following inhibition of angiotensin converting enzyme with 10 μ M captopril ($EC_{50} = 50$ nM; $135 \pm 32\%$, $n = 4$). The depolarization caused by bradykinin, or [des-Arg¹⁰]-kallidin, in captopril and IL-1 β was not enhanced because of a change in sensitivity of treated ganglia to muscarine as the depolarization following activation of m₁ receptors with muscarine (1 μ M) was unaffected (458 ± 82 μ V, $n = 12$) compared to control (506 ± 65 μ V, $n = 8$).

Responses to bradykinin were selectively antagonized by the B₂ receptor antagonist, Hoe 140 but not by the B₁ receptor antagonist, [Leu⁸]-bradykinin₁₋₈ (Table 1). The shift in the concentration-effect curve to bradykinin with increasing concentrations of Hoe 140 was used to generate a Schild plot (Figure 4). The pA₂ value for Hoe 140 in mouse tissue was determined to be 9.65, although the slope of the regression line was significantly greater than unity (slope = 1.88 ± 0.18 , $n = 9$). These data suggest that Hoe 140 was behaving as a non-competitive antagonist in this assay, which precluded the determination of a meaningful antagonist affinity value. Like bradykinin, the depolarization caused by [Lys⁰]-bradykinin was also effectively antagonized by Hoe 140 (3 nM, Figure 4b). As in mouse tissue, Hoe 140 was also an effective antagonist of bradykinin responses in rat ganglia. With an identical antagonist concentration (3 nM) the rightward shift in the concentration-effect curve to bradykinin was used to compare the relative effectiveness of Hoe 140 in mouse and rat tissue (Table 1). Using this method the actions of Hoe 140 were indistinguishable in the two species. However, it should be noted that these estimated pK_A values do not reflect the absolute affinity of the antagonist for the receptors because in this assay Schild analysis revealed that Hoe 140 had non-competitive kinetics.

Discussion

One of the most extensively studied neuronal systems in which the physiological effects of bradykinin receptors have

Figure 3 Concentration-dependent depolarization of mouse isolated superior cervical ganglia by bradykinin and its analogues. (a) Voltage trace from an individual recording following exposure of a ganglion to muscarine (1 μ M, applied for 1 min at arrow) and by increasing concentrations of [Lys⁰]-bradykinin (1 min at 5 min intervals). Vertical calibration bar = 300 μ V. (b) Concentration-effect curves for bradykinin (○), [Lys⁰]-bradykinin (●) and [des-Arg¹⁰]-kallidin (□). Each point represents pooled data from 3 to 6 ganglia. Solid line represents the fit of single site equation to the pooled data. NB the EC₅₀ value for each agonist (Table 1) was determined separately using the geometric mean of the individual logarithmic EC₅₀ values for each ganglion.

been characterized are those of dorsal root ganglia, primarily because these sensory neurones are involved in nociceptive input into the spinal cord. Bradykinin selectively excites a subpopulation of dorsal root ganglia neurones, by mobilizing

Figure 4 Antagonism of responses in mouse ganglia by the selective B_2 receptor antagonist Hoe 140. (a) Schild plot depicting the antagonism of the depolarization caused by bradykinin with increasing concentrations of Hoe 140 after equilibration for 1 h. The concentration of Hoe 140 that was required to shift the agonist EC_{50} value by two, the pA_2 value, was 9.65 (equivalent to 224 pM). However, the slope of the regression line (solid line) was significantly greater than unity (slope = 1.88 ± 0.18) indicating non-competitive antagonism in this assay. (b) Shift in the concentration-effect curve to [Lys⁰]-bradykinin (□) after incubation in Hoe 140 (3 nM for 1 h; ■). Each point represents the mean and s.e.mean from three preparations. Solid lines represent the fit of a single site equation to the pooled data (see Table 1). Note the flattening of the concentration-effect curve in Hoe 140.

intracellular calcium and activation of an inward cation conductance (Perney & Miller, 1989; McGehee *et al.*, 1992; McGuirk & Dolphin, 1992). Bradykinin receptors have also been implicated in mediating some forms of hyperalgesia and inflammation via regulation of the sympathetic nervous system (Levine *et al.*, 1986; Lee *et al.*, 1991). Consequently to investigate the pharmacology of bradykinin receptors on sympathetic neurones we have compared the ability of bradykinin analogues to depolarize rodent superior cervical ganglia, and have also examined the ability of antagonists selective for bradykinin receptor subtypes to inhibit these responses.

Bradykinin depolarized ganglia that had been isolated from both rats and mice. However, the depolarization obtained with bradykinin (3 μ M) was less in mouse versus rat tissue, relative to the maximum depolarization obtained following activation of muscarinic receptors in each species. The weak depolarization observed in mouse tissue was correlated with the 4 fold lower potency of both bradykinin and [Lys⁰]-bradykinin (kallidin) compared to rat tissue under control conditions. Despite this difference, the rank order of agonist potency in both species was consistent with the activation of

B_2 receptors in that bradykinin and [Lys⁰]-bradykinin were equipotent agonists, whereas the B_1 receptor selective agonists, [des-Arg¹⁰]-kallidin and [des-Arg⁹]-bradykinin, were relatively inactive. The selectivity of these agonists was identical to that observed in binding studies using cloned murine B_2 receptors (Hess *et al.*, 1994) and was inconsistent with the ligand binding profile of human B_1 receptors (Menke *et al.*, 1994). Although rodent B_1 receptors have yet to be cloned, the relatively weak response to [des-Arg¹⁰]-kallidin suggested that B_1 receptors contributed only a minor component, if any, to the depolarization of these sympathetic ganglia under control conditions.

Hoe 140 is a potent and selective antagonist of B_2 bradykinin receptors (Hock *et al.*, 1991; Rahleb *et al.*, 1992). Like the binding profile of cloned murine B_2 bradykinin receptors (Hess *et al.*, 1994), nanomolar concentrations of Hoe 140 antagonized the depolarization of isolated superior cervical ganglia caused by both bradykinin and [Lys⁰]-bradykinin. The apparent non-competitive nature of the antagonism by Hoe 140 in this assay may be a consequence of the remarkably high affinity of Hoe 140 (230 pM in binding) for the receptor. High affinity receptor ligands typically have very slow off-rates which can effectively preclude a state of equilibrium being reached during the course of an experiment at low antagonist concentrations. There are alternative explanations for Schild plots in which slopes are greater than unity, including positive co-operativity in the binding of an antagonist to the receptor and/or non-specific binding within the tissue (e.g. Kenakin, 1993), although studies in other tissues indicate that Hoe 140 is capable of binding competitively to B_2 receptors (e.g. Rhaleb *et al.*, 1992).

Angiotensin converting enzyme (kininase II) is one of the principle enzymes that degrade kinins into biologically inactive peptides. This enzyme can be selectively inhibited by the competitive blocker, captopril (reviewed in Horovitz, 1981) and was therefore used to determine whether enzymic degradation influenced the potency or efficacy of exogenously applied bradykinin in isolated superior cervical ganglia. Consistent with the presence of kininase II, captopril caused a 70% increase in the maximum depolarization caused by bradykinin in mouse ganglia and this was accompanied by a >10 fold increase in agonist potency.

Responses to kinins in several tissues including smooth muscle, neurones, and endothelium can also be regulated by cytokines. In rabbit aorta the effects of [des-Arg⁹]-bradykinin, an agonist that is *ca.* 10 fold selective for B_1 over B_2 receptors in binding studies (Hess *et al.*, 1994; Menke *et al.*, 1994), are potentiated by pretreatment with interleukin 1 β (deBlois *et al.*, 1991). This potentiation was blocked by cycloheximide, an inhibitor of protein synthesis, suggesting that it was probably due to an increase in receptor expression. Recently, Schneck and colleagues have also shown that responses to the more selective B_1 agonist, [des-Arg¹⁰]-kallidin are upregulated by epidermal growth factor in cultures of aortic smooth muscle cells, and that this effect is associated with an increase in number of ligand binding sites (Schneck *et al.*, 1994). To investigate whether interleukins also regulate the expression of bradykinin receptors in sympathetic neurones, ganglia were treated with IL-1 β and the responses to bradykinin and [des-Arg¹⁰]-kallidin were examined. In mouse superior cervical ganglia, chronic *in vitro* treatment with IL-1 β selectively potentiated the effect of bradykinin in a temperature-dependent manner, in that the increase was precluded by maintaining ganglia at 4°C during the period of incubation with IL-1 β . In contrast to bradykinin, responses to the B_1 receptor agonist, [des-Arg¹⁰]-kallidin, were unaffected by this treatment under control conditions. However, after inhibition of angiotensin converting enzyme with captopril, the depolarization caused by [des-Arg¹⁰]-kallidin (3 μ M) was also potentiated (from 4% to 35%) by exposure to IL-1 β . Furthermore this effect of [des-Arg¹⁰]-kallidin was selectively blocked by the B_1 receptor antagonist, [Leu⁸]-bradykinin₁₋₈. Thus angiotensin converting enzyme

modulates the effects of exogenously applied kinins in this tissue, and interleukin treatment can potentiate the effects of both B₁- and B₂-selective agonists on sympathetic neurones.

The induction of bradykinin receptor expression by cytokines has been hypothesized to underlie the persistent state of hyperalgesia that accompanies chronic inflammation (Dray & Perkins, 1993). Furthermore sympathetic neurones

are thought to be an important source of prostanoids during inflammation and are involved in a number of pathological hyperalgesic states (Levine *et al.*, 1986; Lee *et al.*, 1991). The ability of interleukin 1 β to potentiate responses to both B₁ and B₂ receptor agonists on sympathetic neurones suggests that one or indeed both of these bradykinin receptor subtypes may be involved.

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